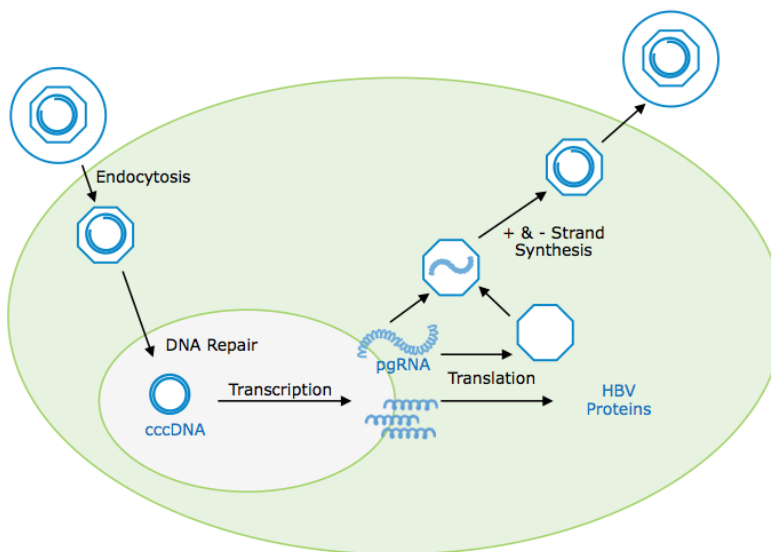


## Hepatitis B Antiviral Drug Development Multi-Marker Screening Assay

### Background

ImQuest BioSciences has developed and qualified a single-plate method to expedite the screening of antiviral agents against multiple markers of hepatitis B virus (HBV) replication.

The hepatitis B virus is one of only a few known non-retroviral viruses that uses reverse transcription as a part of its replication process. After the virus has entered the cell, the partially double-stranded viral DNA is transferred to the nucleus where it is made fully double-stranded and is transformed into covalently-closed circular DNA (cccDNA) that serves as a template for transcription of four mRNAs (**Figure 1**).

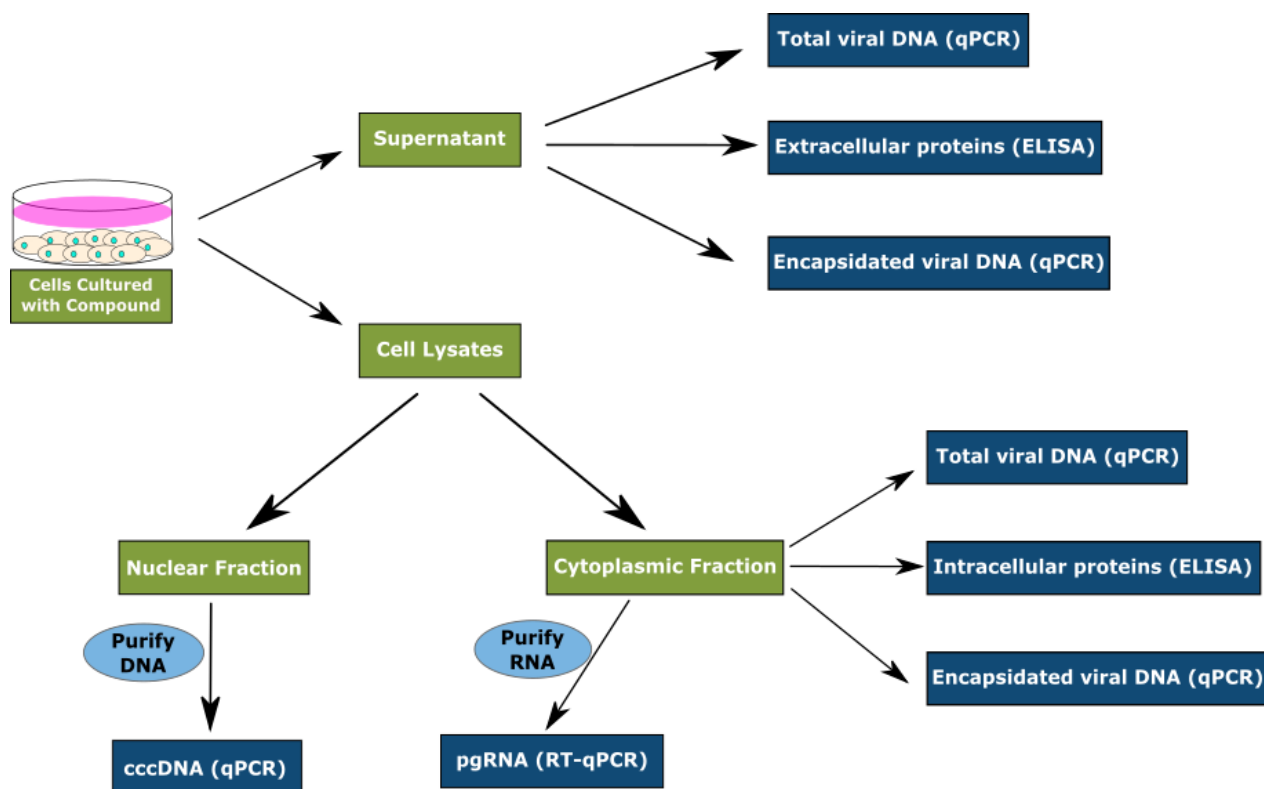


**Figure 1.** Replication of the hepatitis B virus.

The largest mRNA, the pre-genomic RNA (or pgRNA), is translated to produce the hepatitis B core antigen (HBcAg, or nucleocapsid), the soluble and secreted HBeAg, and the polymerase. The pgRNA is also used to make new copies of the viral genome by reverse transcription. The other mRNAs are translated to produce the viral envelope proteins and the HBV X protein. The antiviral activities of the nucleoside reverse transcriptase inhibitors 2',3'-dideoxy-3'-thiacytidine (3TC) and tenofovir disoproxil fumarate (TDF) on HBV replication in Hep AD38 and HepG2.2.15 cells were evaluated as a means of development and qualification of a 96-well plate assay.

**Assay** ImQuest Biosciences has developed a methodology using Hep AD38 and HepG2.2.15 cells. HepG2.2.15 cells are derived from the human hepatoblastoma cell line HepG2 by stable transfection with full length HBV plasmid. Hep AD38 cells express proteins, RNA, and DNA intermediates characteristic of HBV replication. The Hep AD38 cells are derived from HepG2 stably transfected with an expression vector in which HBV replication is regulated by tetracycline.

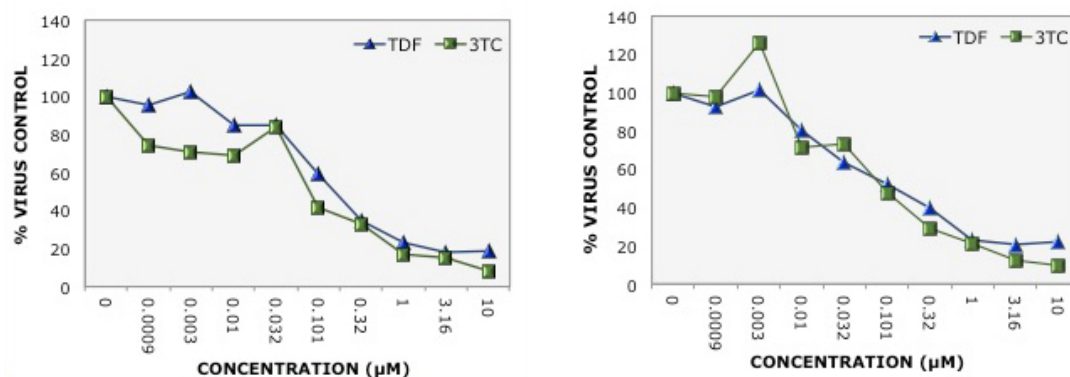
Cells are seeded into 96-well plates and treated with serial half-log dilutions of TDF or 3TC for six days. Cell culture supernatants are then collected for quantification of extracellular HBV markers. The cells are lysed and fractionated into nuclear and cytoplasmic components for evaluation of intracellular markers of viral replication (**Figure 2**).



**Figure 2.** Procedure for analysis of multiple HBV markers of replication from a 96-well plate.

## Results *Viral DNA Synthesis*

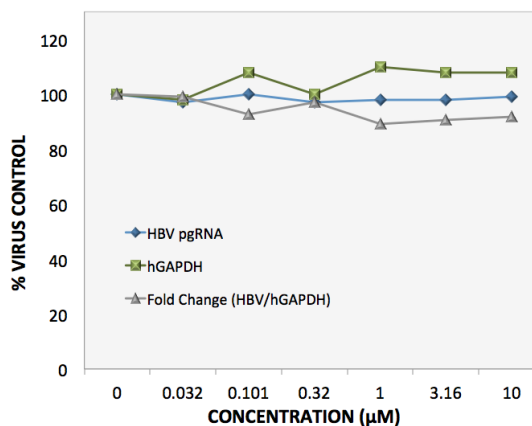
There was a dose-dependent reduction in extracellular HBV DNA based on evaluation of the effect of TDF and 3TC on HBV replication in both HepG2.2.15 and Hep AD38 cells (**Figure 3**). The cell culture supernatants were diluted 1:10 in PCR dilution buffer. The samples were boiled and amplified with specific primers and TaqMan probes.



**Figure 3.** HBV replication in HepG2.2.15 (left) and Hep AD38 (right) cells treated with TDF or 3TC by quantification of viral DNA in the cell culture supernatant.

## *HBV pgRNA Transcription*

There was no reduction of pgRNA transcription by 3TC, based on the evaluation of the effect of 3TC on the synthesis of pgRNA in Hep AD38 cells (**Figure 4**). To determine the effect of 3TC, nucleic acids (specifically extracted from the cytoplasmic fraction) were treated with DNase prior to RT-qPCR with HBV specific primers and TaqMan probes.



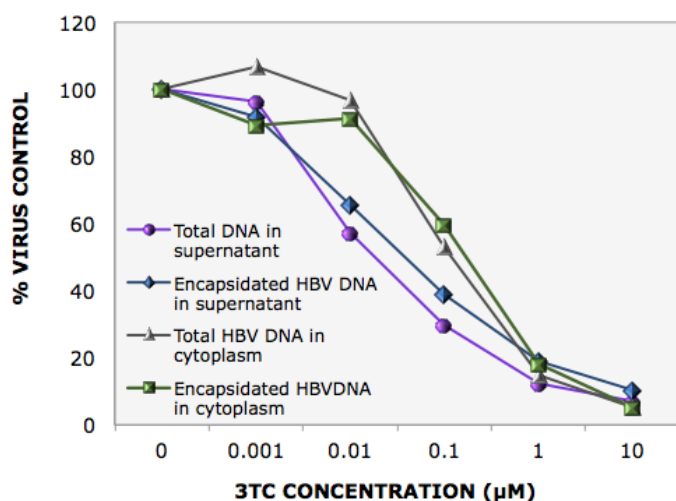
**Figure 4.** Analysis of HBV pgRNA accumulation in Hep AD38 cells treated with 3TC.

## Encapsidated Viral DNA Production

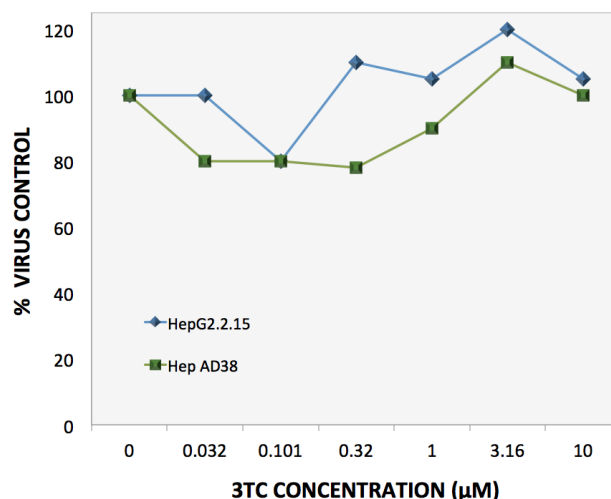
The effect of 3TC on viral DNA replication in Hep AD38 cells was dose-dependent. Cell culture supernatant and cytoplasmic fractions from cell lysates were treated with micrococcal nuclease to digest unencapsidated nucleic acids. The enzyme was then inactivated with EGTA. Nuclease-treated samples were boiled in PCR buffer and subjected to qPCR with HBV primers and probe (**Figure 5**).

## cccDNA Accumulation

Neither cell lines demonstrated a dose-dependent reduction in accumulation of cccDNA upon treatment with a 10-fold dilution scheme of 3TC. This lack of inhibitory activity of 3TC on cccDNA accumulation is anticipated, due to the fact that 3TC acts at the reverse transcription event, which occurs downstream to the cccDNA accumulation (**Figure 6**).



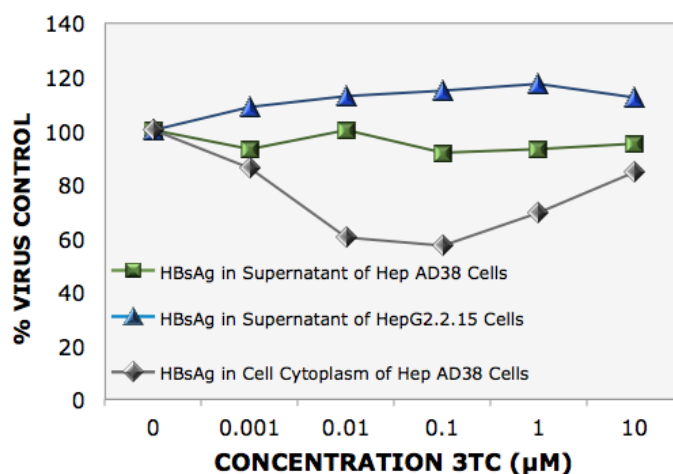
**Figure 5.** Analysis of HBV intracellular and extracellular encapsidated DNA and whole viral DNA replication in 3TC-treated Hep AD38 cells.



**Figure 6.** cccDNA in the nuclear fraction from Hep AD38 and HepG2.2.15 cells treated with 3TC.

### HBV Antigen Production

To evaluate the effect of 3TC on synthesis of HBV antigens, cell culture supernatant and cell cytoplasm were analyzed for the presence of HBsAg and HBcAg by ELISA. Reduction of HBsAg and HBcAg by 3TC was not observed at the highest test concentration (10  $\mu$ M) (**Figure 7**).



**Figure 7.** HBV intracellular and extracellular proteins in Hep AD38 and HepG2.2.15 cells treated with 3TC.

### Antiviral Drug Efficacy

EC<sub>50</sub> values for inhibition of extracellular HBV DNA accumulation were comparable in the two lines; EC<sub>50</sub> values for inhibition of extracellular DNA were lower than those for cytoplasmic DNA in both cell lines (**Table 1**).

Markers	Hep AD38		Hep G2.2.15	
	3TC	TDF	3TC	TDF
Total HBV DNA in supernatant	0.09	0.126	0.081	0.157
Encapsidated HBV DNA in supernatant	0.04	---	---	---
Total HBV DNA in cytoplasm	0.12	---	0.35	---
Encapsidated HBV DNA in cytoplasm	0.17	---	---	---
cccDNA	>10.0	---	>10.0	---
HBsAg in cytoplasm	>10.0	---	>10.0	---
HBcAg in cytoplasm	>10.0	---	---	---

**Table 1.** EC<sub>50</sub> values for inhibition in the panel of HBV markers.

## **Summary**    *A broad spectrum method to screen multiple HBV markers*

ImQuest Biosciences has developed a screening method to test the effect of antiviral agents on multiple markers of HBV replication from a 96-well plate. The method was developed and qualified by evaluating the effect of reverse transcriptase inhibitors on multiple HBV markers in both HepG2.2.15 and Hep AD38 cells. Results demonstrate a dose-dependent 3TC-mediated reduction in extracellular HBV DNA and cytoplasmic HBV DNA, consistent with the inhibition of HBV replication by an inhibitor of reverse transcription.

### ***About ImQuest Biosciences...***

ImQuest BioSciences focuses on preclinical contract research and development that evaluates the potential of new and novel pharmaceutical products.

We specialize in the development of drugs, vaccines, and biologic products for the treatment and prevention of infectious disease, cancer, and inflammatory disease.

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